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SYMPOSIUM REVIEW

Deconstructing calsequestrin. Complex buffering in the calcium store of skeletal muscle

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Since its discovery in 1971, calsequestrin has been recognized as the main Ca^{2+} binding protein inside the sarcoplasmic reticulum (SR), the organelle that stores and upon demand mobilizes Ca^{2+} for contractile activation of muscle. This article reviews the potential roles of calsequestrin in excitation–contraction coupling of skeletal muscle. It first considers the quantitative demands for a structure that binds Ca^{2+} inside the SR in view of the amounts of the ion that must be mobilized to elicit muscle contraction. It briefly discusses existing evidence, largely gathered in cardiac muscle, of two roles for calsequestrin: as Ca^{2+} reservoir and as modulator of the activity of Ca^{2+} release channels, and then considers the results of an incipient body of work that manipulates the cellular endowment of calsequestrin. The observations include evidence that both the Ca^{2+} buffering capacity of calsequestrin in solution and that of the SR in intact cells decay as the free Ca^{2+} concentration is lowered. Together with puzzling observations of increase of Ca^{2+} inside the SR, in cells or vesicular fractions, upon activation of Ca^{2+} release, this is interpreted as evidence that the Ca^{2+} buffering in the SR is non-linear, and is optimized for support of Ca^{2+} release at the physiological levels of SR Ca^{2+} concentration. Such non-linearity of buffering is qualitatively explained by a speculation that puts together ideas first proposed by others. The speculation pictures calsequestrin polymers as 'wires' that both bind Ca^{2+} and efficiently deliver it near the release channels. In spite of the kinetic changes, the functional studies reveal that cells devoid of calsequestrin are still capable of releasing large amounts of Ca^{2+} into the myoplasm, consistent with the long term viability and apparent good health of mice engineered for calsequestrin ablation. The experiments therefore suggest that other molecules are capable of providing sites for reversible binding of large amounts of Ca^{2+} inside the sarcoplasmic reticulum.

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Skeletal muscle has a large concentration of Ca^{2+} bound inside stores

In skeletal fast twitch muscle cells of amphibians and some mammals studied in detail, more than 200 μmol of Ca^{2+} per litre of myoplasm is rapidly released from the sarcoplasmic reticulum (SR) after an action potential, to start the process required for contraction (Pape *et al.* 1993; Baylor & Hollingworth, 2003). This amount appears to be between 10 and 17% of the total Ca^{2+} that can be released from the storage organelle (Pape *et al.* 1993; Pizarro & Rios, 2004; Launikonis *et al.* 2006; Rudolf *et al.* 2006), which for fast-twitch fibres at rest (in a variety of preparations) is estimated at between 1 and 5 mmol per litre of myoplasm (e.g. Schneider *et al.* 1987; Jong *et al.* 1993; Fryer & Stephenson 1996; Owen *et al.* 1997). These estimates can be converted to concentration in the volume of the fibre considering that the accessible myoplasm occupies approximately 0.7 of the muscle volume.

Because most Ca^{2+} appears to be released from the terminal cisternae (TC) of the SR, compartments that

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occupy only ∼2% of the total cell volume in the best studied mammalian species (Eisenberg, 1984) and have therefore some 35 times less volume than the accessible myoplasm, the total concentration of calcium in TC at rest must be between 35 and 175 mM.

The concentration of free Ca^{2+} inside the resting SR of skeletal muscle was initially estimated at 1 or 2 mM based on indirect considerations or comparisons with other tissues (e.g Chen *et al.* 1996). It has now been measured, using two different ratiometric dyes, at 0.35 mM in the frog (Launikonis *et al.* 2005) or 0.31 mM in the mouse (Rudolf *et al.* 2006). Based on these figures, the buffering power of the TC, ratio between total and free $[Ca^{2+}]$, must be between 100 and 500. Therefore, a Ca^{2+} binding structure of large capacity must exist inside the SR. Because Ca^{2+} must be available for rapid release, the storage sites must have a high unbinding rate, which constrains the affinity to be relatively low, far from saturated at the physiological $[Ca^{2+}]_{SR}$.

Calsequestrin may provide the binding sites and additional regulation

Calsequestrin (MacLennan & Wong, 1971; referred to as Casq) satisfies some of the requisites for being the main provider of these sites. In the adult, mammalian fast-twitch fibres contain only isoform Casq1 (Sacchetto *et al.* 1993), while in slow-twitch fibres the 'cardiac' isoform, Casq2, is coexpressed with Casq1 at a 1 : 3 ratio (Damiani *et al.* 1990; Sacchetto *et al.* 1993; Murphy *et al.* 2009). Volpe & Simon (1991) measured the concentration of Casq in frog muscle and inferred a maximal binding capacity of 0.67 mmol per litre of available myoplasmic volume. This is at the low end of the range of releasable Ca^{2+} estimated by functional studies, and the discrepancy becomes major if one considers that Casq must not be saturated of Ca^{2+} at rest. Likewise, Leberer & Pette (1986) measured the Ca^{2+} binding capacity of Casq in rabbit muscle at less than 1 mmol per litre of myoplasm. The deficit of the early estimates has been reconciled in recent measurements by Murphy *et al.* (2009) in fast twitch muscle of the rat yielding 36 μmol Casq1 per litre of fibre volume, which results in a Ca^{2+} binding capacity of 4.1 mM in terms of accessible myoplasmic volume. These authors also confirm the difference between maximal storage capacity and resting SR calcium content, which Fryer & Stephenson (1996) showed to be about 30% of the maximum in fast-twitch fibres. Finally, Casq1 appears to have the requisite low affinity (reported at $(1\ mM)^{-1}$; Park *et al.* 2003; Volpe & Simon, 1991; but note that this property depends on the aggregation state of the protein, as described below).

In addition to this reversible buffer role, there is evidence that Casq functions as an intra-SR Ca^{2+} 'sensor', that is, a mediator of the observed modulatory effects of $[Ca^{2+}]_{SR}$ on the activity of Ca^{2+} release channels. This idea is widely accepted in cardiac muscle, where $[Ca^{2+}]_{SR}$ is reported to exert a two-way modulation: its increase promotes release channel opening (Shannon *et al.* 2000; Györke *et al.* 2002), while depletion of SR, due to Ca^{2+} release, is thought to be a major agonist of the channel closing that terminates Ca^{2+} release in Ca^{2+} sparks and cell-wide Ca^{2+} release in physiological conditions (Cheng & Lederer, 2008; Ríos *et al.* 2006; Sobie *et al.* 2002; Terentyev *et al.* 2002).

Cardiac calsequestrin is believed to participate in this modulation both by virtue of its buffer properties (which determine the evolution of $[Ca^{2+}]_{SR}$) and by altering the tendency of the release channel to open, an effect that requires the SR protein triadin, presumably as a physical linker between RyR and Casq (Györke *et al.* 2004). These effects and mechanisms, as well as their incidence in the pathogenesis of diseases affecting cardiac rhythm, are discussed by others in this volume (see also Terentyev *et al.* 2003; Knollmann *et al.* 2006; Györke & Terentyev, 2008; Terentyev *et al.* 2008; Valle *et al.* 2008; Liu *et al.* 2009).

Manipulation of Casq1/2 produces contradictory results in skeletal muscle

By contrast, the functions of Casq1 in skeletal muscle have not been fully identified. As mentioned above, its role as main Ca^{2+} storage is consistent with the measurements of content and its variations in a recent study of rat muscle (Murphy *et al.* 2009) and supported in a study of a Casq1-null mouse (Paolini *et al.* 2007), in which the peak Ca^{2+} transient elicited by an electric stimulation decreases significantly (a change of ∼35% in fura-2 ratio) and the integral of tension over time in a caffeine contracture decreases by about 80% compared with the wild-type. On the other hand Wang *et al.* (2006; see also Meissner *et al.* 2009) found that nearly complete removal of Casq1 did not affect the amount of releasable Ca^{2+} in C_2C_{12} skeletal myotubes, and the Casq1-null mouse studied by Paolini *et al.* (2007) was capable of close to normal contractile function and exhibited increased resistance to fatigue. Our own work, still in progress, is in line with the above results. It reveals only minor decrease in releasable Ca^{2+} in muscle fibres of a mouse devoid of calsequestrins 1 and 2, as well as a small increase in releasable Ca^{2+} in adult mouse muscle overexpressing Casq1 by several times the native content. The cardiac field is also less than unanimous in this regard: the study of a Casq2-null mouse revealed a reduction of only 14% of its releasable Ca^{2+}, a reduction that in fact was entirely attributable to changes in SR Ca^{2+} leak rather than buffering capacity (Knollmann *et al.* 2006; Knollmann, 2009).

The results obtained with skeletal muscle are also ambiguous as regards the putative modulation of the release channels by Casq1 or 2. Studying RyR channels

from SR fractions reconstituted in bilayers, Wang *et al.* (2006) found that Casq1 added to the lumenal or *trans* side enhanced the activity of channels derived from C_2C_{12} muscle myotubes silenced for both Casq isoforms. Beard *et al.* (2002) also found an effect, but it was in this case a reduction in open probability of rabbit SR channels with Casq removed by high salt. Additionally, a 'malignant hyperthermia-like' syndrome, reminiscent of the instability caused by Casq2 changes in the heart, was reported for the Casq1-null mouse muscle (Dainese *et al.* 2009; Protasi, 2009). In view of the contradictions among these and other studies, it seems fair to state that the details and significance of RyR modulation by Casq1 remain to be established.

The location and aggregation of calsequestrin suggest specific functions

As evidenced in Fig. 1 (unpublished images kindly provided by S. Boncompagni and C. Franzini-Armstrong) Casq1 forms an intricate network in skeletal SR of mice (Fig. 1*A*), toadfish (Fig. 1*B*) and other species. The electron microscopic features are believed to result from polymerization that is predominantly one dimensional, but frequently ramifies, leading to the characteristic 'can of worms' appearance. One notable feature is the presence of connecting tendrils (arrows), which link the tangle of linear and ramified segments to the lumenal side of the junctional membrane, near individual RyRs. In both cases the tendrils are singular on the channel side, but ramified, like a bouquet or a tree, on the lumenal side. This connection is known to be mediated by triadin and junctin (Guo *et al.* 1994; Zhang *et al.* 1997; Tijskens *et al.* 2003) but there may be other connecting molecules (Treves 2009). The detailed, stoichiometric linking of a Casq 'tree' converging on the channel suggests either an allosteric interaction for channel modulation, or a device for delivery of calcium near the lumenal channel mouth, as proposed by MacLennan & Reithmeier (1998) and Park *et al.* (2003, 2004).

Polymerization of calsequestrin has consequences for calcium buffering

Ca^{2+} binding to Casq is known to affect its conformation and reactivity, reducing its Stokes radius, hydrophobicity and susceptibility to trypsin digestion (Ikemoto *et al.* 1974; Mitchell *et al.* 1988; He *et al.* 1993; Wang *et al.* 1998). All these effects are interpreted as consequences of the stabilization by Ca^{2+} of an electronegative core in a folded, compact conformation (Park *et al.* 2003).

Nearly two-thirds of the negatively charged residues of Casq reside in its C-terminal segment, which suggests that the C terminus is the Ca^{2+}-binding region of the molecule. However, the binding capacity of the isolated C-terminal peptide is much lower than that of the full protein. High capacity binding requires the protein to be in aggregates, formed when both Casq and Ca^{2+} are at high concentrations (Aaron *et al.* 1984; Tanaka *et al.* 1986).

C. Kang and coworkers determined binding capacities of Casq1 and 2 by atomic absorption spectroscopy, while monitoring circular dichroism, light scattering and fluorescence (Park *et al.* 2004). As shown in Fig. 2, the binding curves of both Casq1 and 2 feature discrete changes in curvature that clearly separate from simple binding. The parallel measurements of physical properties allowed the authors to associate these transitions to successive steps in polymerization (first forming dimers, then tetramers, etc.). C. Kang (personal communication) suggests that the observed creation of sites upon polymerization reflects the advantage that two linear peptide segments with acidic residues have in coordinating Ca^{2+}, by comparison with a single peptide chain, which needs to fold – and pay the consequent thermodynamic penalty – if it is to individually bind the ion. This idea is powerful, as it also explains the complementary observation, that Ca^{2+} stabilizes the polymer, and decrease in its concentration results in restoration of the monomericstate.

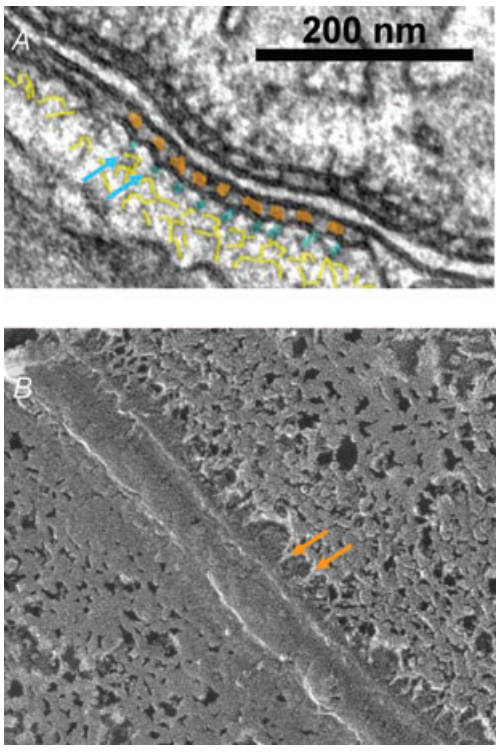

Figure 1. The calsequestrin network in skeletal muscle
A, electron microscopic image from mouse FDB. Colorization by hand, according to the following code: yellow, Casq network; blue-green, 'tendrils' connecting calsequestrin network to junctional SR; orange, RyRs. *B*, deep etch image from toadfish muscle. Arrows indicate tendrils. Unpublished images of C. Franzini-Armstrong and S. Boncompagni.

In conclusion, the Ca^{2+} binding capacity of Casq increases with $[Ca^{2+}]$; the increase occurs in steps, concomitant with early stages of the formation of a linear polymer. As discussed below, these properties find an excellent correlate in the strongly non-linear properties of Ca^{2+} buffering inside the SR.

Evidences of non-linear buffering inside the SR

The first indication of 'anomalies' in the Ca^{2+} binding properties of Casq was noted by Ikemoto *et al.* (1991) who showed that $[Ca^{2+}]$ initially increased inside SR vesicles stimulated to release Ca^{2+} through its channels, an increase that was dependent on the presence of Casq and therefore attributed to a dissociation of Ca^{2+} from Casq inside the store. From what we now know about Casq binding properties, the dissociation was probably caused by an initial release of Ca^{2+} through RyR channels, with consequent decay in lumenal $[Ca^{2+}]$ leading to Casq depolymerization.

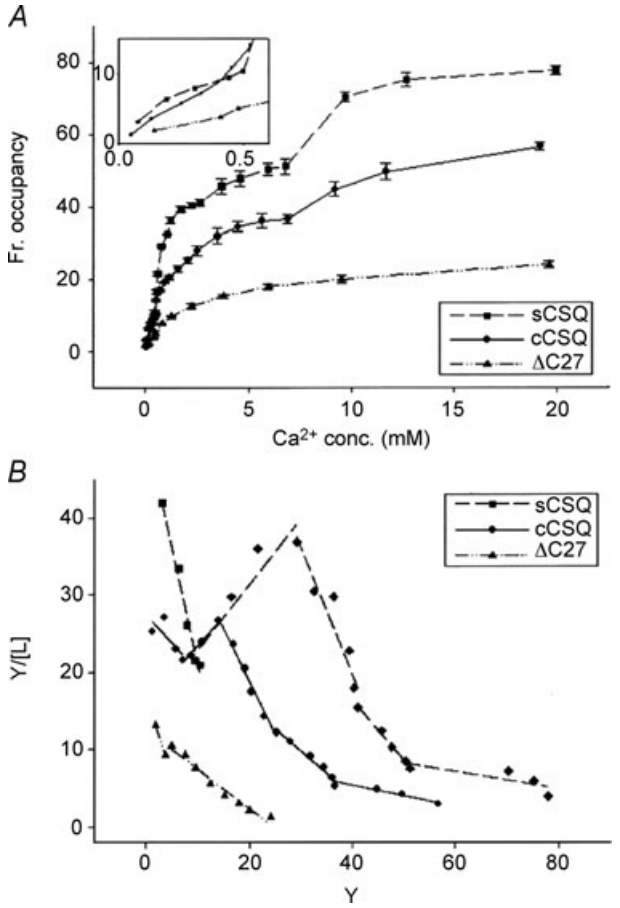

Figure 2. Ca^{2+} binding properties of calsequestrins in aqueous solution
A, fractional occupancy (Y = [bound Ca^{2+}]/[total Casq]) was plotted against unbound ligand concentration. Inset: magnified view of the Ca^{2+} range 0–0.7 mM. *B*, scatchard-type plot of the same data, which shows that the dissociation constant (slope) varies according to the degree of ligand binding. sCSQ represents Casq1, cCSQ is Casq2 and ΔC27 a mutant with the last 27 residues deleted. Note that the mutant shows no transitions in binding capacity. Reproduced from Park *et al.* (2004).

Pape *et al.* (2007) used the absorption changes of tetramethylmurexide inside frog muscle SR to determine simultaneously the evolution of its free and total Ca^{2+} concentrations (respectively $[Ca^{2+}]_{SR}$ and $[Ca]_{T,SR}$) during Ca^{2+} release. Their results were consistent with a substantial decrease of SR Ca^{2+} buffering (both 'chord' buffering power $B = [Ca]_{T,SR}/[Ca^{2+}]_{SR}$ and 'slope' buffering power $b = d[Ca]_{T,SR}/d[Ca^{2+}]_{SR}$) upon depletion of Ca^{2+} in the SR. This decay was attributed to concentration-dependent changes in buffering by Casq although no separate evidence was presented for the involvement of Casq in these effects.

Our laboratory first encountered evidences of non-linear buffering when we used Shifted Excitation and Emission Ratioing (SEER) (Launikonis *et al.* 2005) of the fluorescence of mag-indo 1 trapped inside the SR of frog muscle to image the local intra-SR depletion of Ca^{2+} associated with a Ca^{2+} spark. This image, represented in Fig. 3 together with the simultaneously recorded spark, was named the 'skrap' while being sought, as we anticipated it would have close to mirror symmetry with the spark. However, skraps were anything but symmetrical with sparks. As shown in profile in Fig. 3*B*, the amplitude was small, consistent with other evidence that skeletal SR does not deplete easily (review, Rios *et al.* 2006). But the most surprising feature was the temporal lag of the skrap. Indeed, the skrap reaches its nadir (minimum of $[Ca^{2+}]_{SR}$) tens of milliseconds later than the peak of the spark, which is believed to mark the end of the underlying release flux – the closing of the channels. This implies that Ca^{2+} depletion continues to develop long after the release channels have closed, an apparent breach of mass conservation. The result led us to second the suggestion of MacLennan & Reithmeier (1998) and Park *et al.* (2004) that there is an immediate source for releasable Ca^{2+} other than the lumenal solution. Specifically, polymerized calsequestrin could bear Ca^{2+} in large quantities and deliver it effectively to the lumenal mouth of the channel, constituting the immediate or proximate source (Launikonis *et al.* 2006).

An anomaly of a different sort, looking like calcium mass creation at a cell-wide level, was reported in the same article (Launikonis *et al.* 2006). The observation was made while applying the same imaging techniques to membrane-permeabilized frog fibres subjected to a mild stimulation of their RyRs. The unexpected result, shown in Fig. 4, was an increase in measured $[Ca^{2+}]_{SR}$, which occurred at the same time as Ca^{2+} was being released to the myoplasm. This exceedingly anomalous behaviour again could be explained assuming a sudden disassembly of Casq polymers, whereby the ensuing loss of buffer

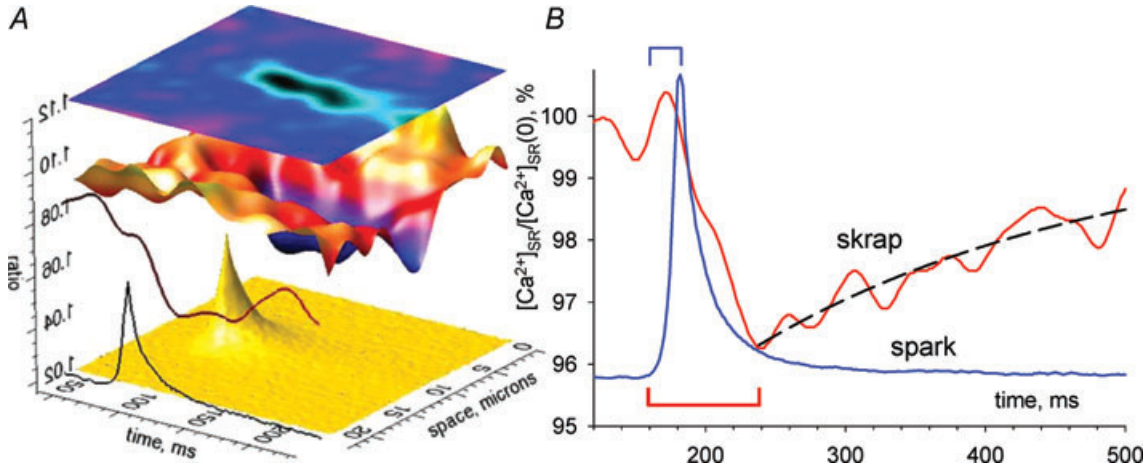

Figure 3. 'Skraps' of depletion inside the skeletal SR
A, surface representation of an average of 6000 sparks (yellow) in line-scans of fluorescence of fluo-4 inside membrane-permeabilized frog semitendinosus muscle fibres. Above the spark, in 'rainbow' palette, is the average of simultaneously recorded skraps, from SEER ratio images of fluorescence of mag-indo 1 inside the SR. *B*, evolution of spark (blue) and skrap (red) amplitude at their spatial maxima. The time to nadir of the skrap (red bracket) outlasts by ~60 ms the time to peak of the spark (blue bracket). This delay, confirmed with other dyes, suggests the existence of a 'proximate source' for Ca^{2+} release that is different from the free lumenal SR Ca^{2+} monitored by mag-indo 1. Modified from Launikonis *et al.* (2006).

capacity leads to an intra-SR release of Ca^{2+}, much as observed by Ikemoto *et al.* (1991) in SR vesicles. A novelty in our images was that the intra-SR increase in $[Ca^{2+}]$ was secondary to the early stages of Ca^{2+} release into the myoplasm, and therefore could be explained as the consequence of depolymerization of the storage protein, induced by Ca^{2+} depletion.

The SR evacuability measures Ca^{2+} permeability and Ca^{2+} buffering

Royer *et al.* (2008) examined the time course of flux in mouse muscle cells subjected to long-lasting activation of Ca^{2+} release by membrane depolarization. While the early stages (within 100 ms) of flux of Ca^{2+} release had properties similar to those described in previous studies for frogs and mammals, including an early peak followed by decay to a 2–4 times lower level, this study looked for the first time at the evolution beyond 100 ms in mouse muscle. The evolution, shown in Fig. 5, is remarkable for the fact that the lower level reached after the peak (marked *QS* in the figure) is only sustained briefly, and followed by decay to a much lower level *S*. This decay is not exponential. The time course can be described as a 'shoulder'. The qualitative features were shown to have a quantitative correlate in an index constructed from the flux by the algorithm:

$$NFRC \equiv -\frac{\mathrm{d}\dot{R}/\mathrm{d}t}{\dot{R}_{\mathrm{net}}}$$

where $\dot{R}$ is the Ca^{2+} release flux and $\dot{R}_{\mathrm{net}}$ is the net flux, difference of $\dot{R}$ and rate of re-sequestration into the SR by the SERCA pump. The *NFRC* (normalized flux rate of change) was shown to be approximately equal to:

$$E \equiv \frac{\varphi P}{B}$$

where P is the SR release permeability for Ca^{2+}, B is the SR buffering power (defined earlier) and φ is a geometric factor.

E (named the 'evacuability') is a useful index: if P and B are constant, the decay of $\dot{R}$ will be exponential, with E as rate constant. E is therefore a generalization of the exponential rate constant, applicable to cases where the decay of flux is not exponential. At the time the decay has its shoulder, the evacuability is low and then increases as the SR is depleted of Ca^{2+}.

The increase in E with depletion may imply an increase in P, a decrease in B, or both. Experimental evidences of both changes exist. Increases in P were inferred observing that the ratio $\dot{R}/[\mathrm{Ca}]_{\mathrm{T,SR}}$ increases as SR content decreases (Pape *et al.* 1995; Pape & Carrier, 1998; Fénélon & Pape, 2002; Pizarro & Ríos 2004). Because changes in $\dot{R}/[\mathrm{Ca}]_{\mathrm{T,SR}}$ could also reflect changes in B, however, this evidence is ambiguous. On the other hand, the fact that the rate of decay of $\dot{R}$ from its initial peak is reduced by both SR Ca^{2+} depletion (Pape *et al.* 1995; Pape & Carrier, 1998) and the presence of fast buffers in the cytosol (as first shown by Baylor & Hollingworth, 1988), supports the conclusion that P increases upon Ca^{2+} depletion due to decrease of the inactivation of channels by released Ca^{2+} (see also Pizarro & Ríos, 2004; Ríos *et al.* 2008). Finally, and as mentioned above, a decrease in B upon depletion of Ca^{2+} in the SR of

frog muscle has been demonstrated by Pape *et al.* (2007) with a direct measurement of $[Ca^{2+}]_{SR}$.

The disparate group of observations summarized above, preliminarily confirmed in ongoing work of our laboratory, may be described collectively as non-linear buffering in the SR. They imply that Casq is an unusual Ca^{2+} buffer, which is likely to work in unusual ways. In the following we illustrate and develop a set of ideas, admittedly speculative, that may help understand the unusual features of Ca^{2+} buffering in the SR. Most of the ideas were first proposed by MacLennan & Reithmeier (1998) and Kang and coworkers (Park *et al.* 2003, 2004 and personal communications); some were previously discussed by Launikonis *et al.* (2006).

How calsequestrin might buffer Ca^{2+} and mediate its delivery

The model is represented schematically in Fig. 6*A*. It incorporates the general structure revealed by electron microscopic images near the channel. It represents the 'trees' or tendrils that connect the channels with the TC-wide Casq network, which is not represented. The polymeric structure is drawn as linear for simplicity, but ramifications are known to occur. Following Park *et al.* (2003) polymerization is shown as taking place between 'like' sides of the protomers (that is, front-to-front, or back-to-back), but see Gatti *et al.* (2001) for an alternative view.

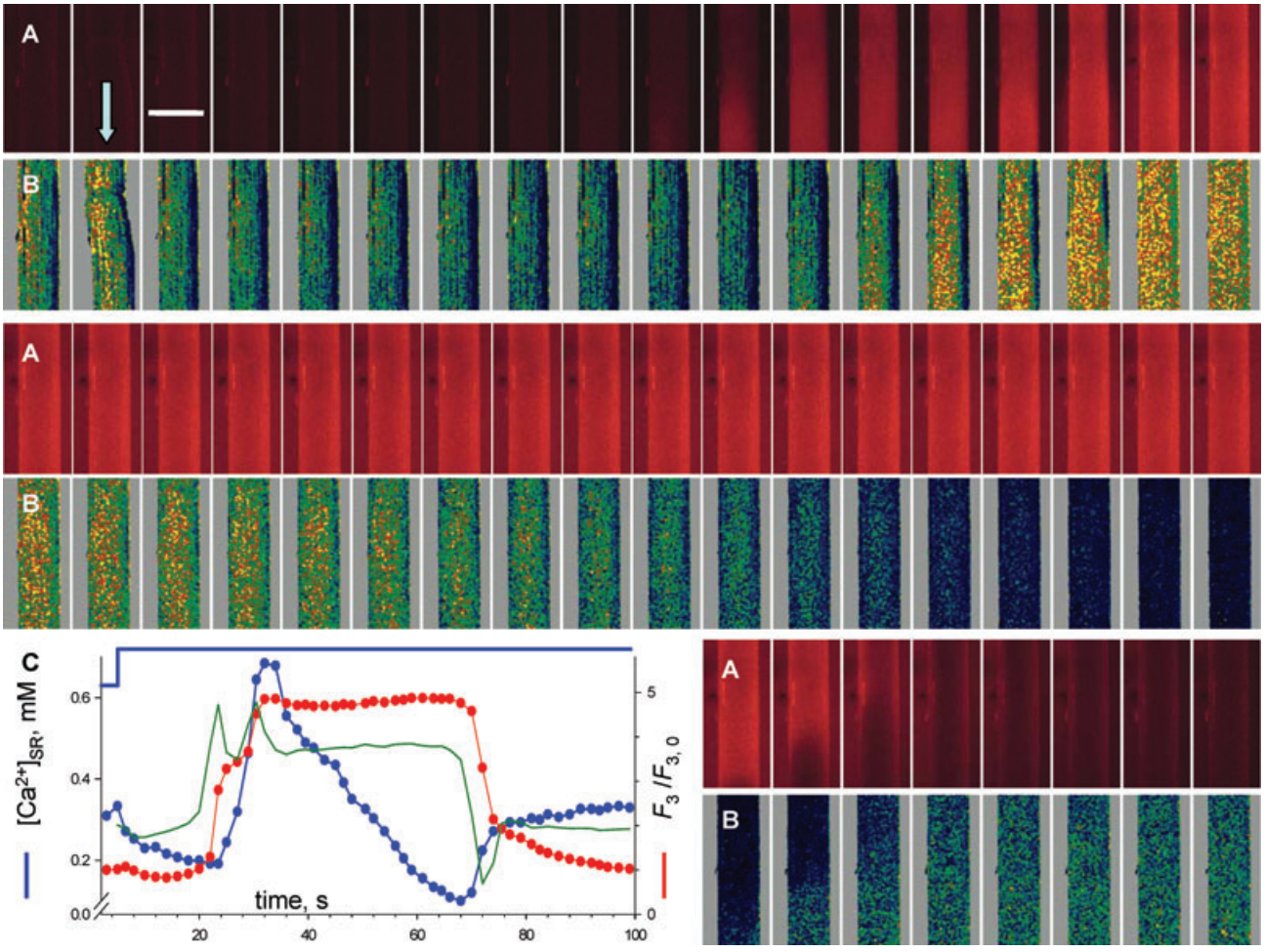

Figure 4. Intra-SR release of Ca^{2+}. Simultaneous imaging of cytosolic and SR $[Ca^{2+}]$ in a frog muscle fibre with permeabilized plasma membrane
A, *xy* scans of fluorescence F_3 of rhod-2 in cytosol. *B*, $[Ca^{2+}]_{SR}$ derived from ratios of simultaneous SEER images of mag-indo 1 inside SR. *C*, image averages of $F_3/F_{3,0}$ (red) and $[Ca^{2+}]_{SR}$ (blue). During acquisition of the 2nd set (arrow in *A*), the solution was changed to one with low Mg^{2+} (upper blue trace in *C*), eliciting Ca^{2+} release. An increase in $[Ca^{2+}]_{SR}$ followed shortly after the beginning of the cytosolic transient. Green, time course of net Ca^{2+} release flux. The second peak of release flux, accompanied by a peak of intra-SR release (blue), again implies input from an additional source, presumably calsequestrin. Republished from Launikonis *et al.* (2006).

In these polymers bound Ca^{2+} occupies an adsorption layer, depicted as peripheral to the polymers, where it is free to move. This feature is complemented in the original proposals (MacLennan & Reithmeier, 1998; Park *et al.* 2003) with the concept that diffusion along this adsorption layer is a means for efficient delivery of Ca^{2+} near the channel mouth. The concept is based in the proposal by Adam & Delbrück (1968) that ligand binding to cell surface receptors is enhanced by the two-dimensional diffusion that follows membrane adsorption of ligands. Direct experimental evidence of such 'diffusion enhancement by reduction of dimensionality' has been found in the case of cations bound to planar charged surfaces of clays, in studies of materials adequate for the storage of radioactive waste (Rotenberg *et al.* 2007 and references therein). Quantitative models of this effect suggest enhancement factors of 100 or more by reduction to two dimensions (DeLisi, 1980; Axelrod & Wang, 1994). Diffusion along linear polymers of calsequestrin, which is better modelled as occurring in one dimension, would involve an additional reduction of dimensionality. A version of the theory especially applicable to the case at hand is the 'perfect sink' model of delivery of ions to an open channel (Berg & Purcell, 1977). In any case, it is not possible to predict how much advantage this will provide in the case at hand; the answer will depend on many details still unknown, including the geometry, ion concentration and free energy profile (which will determine the effective Ca^{2+} diffusion coefficient) of the adsorbed layer.

A final aspect of the model, which follows trivially from the observed dependency of polymerization on $[Ca^{2+}]$ increase, is the notion that depletion results in depolymerization (as depicted in the last panels of Fig. 6).

It additionally helps understand the decrease in buffering power upon depletion reported for frog muscle (Pape *et al.* 2007) as well as our own observations of increase in E (interpreted as due to decrease in B) with depletion in the WT muscle.

The assumed interactions of calsequestrin are allosteric, in the sense that a ligand, Ca^{2+}, allosterically alters the conformation of the protomers, and their tendency to polymerize. A characteristic of allosteric proteins is their ability to engage in concerted changes. Launikonis *et al.* (2006) proposed that an initial descent in lumenal $[Ca^{2+}]$ may result in a phase transition-like disintegration of the polymeric network, which may account for the observation of 'intra-SR release' upon channel activation (Ikemoto *et al.* 1991; Launikonis *et al.* 2006). Likewise, Ca^{2+} lost from the calsequestrin network near channels producing a spark must be restored by Ca^{2+} from the SR lumen, which may explain the lag between Ca^{2+} release in a spark and the measured depletion skrap.

The possibility of concerted changes in the aggregation state of calsequestrin suggests that altering calsequestrin properties (by genetic manipulations currently pursued by several laboratories) may result in non-trivial outcomes as well. For example reduction of Casq1 endowment by silencing of its gene may change the stability of the polymeric network, resulting in sudden changes in buffering power B (the mechanism depicted in Fig. 6*D*). A simpler mechanism for discrete transitions follows from the assumption that the tendrils connecting the Casq

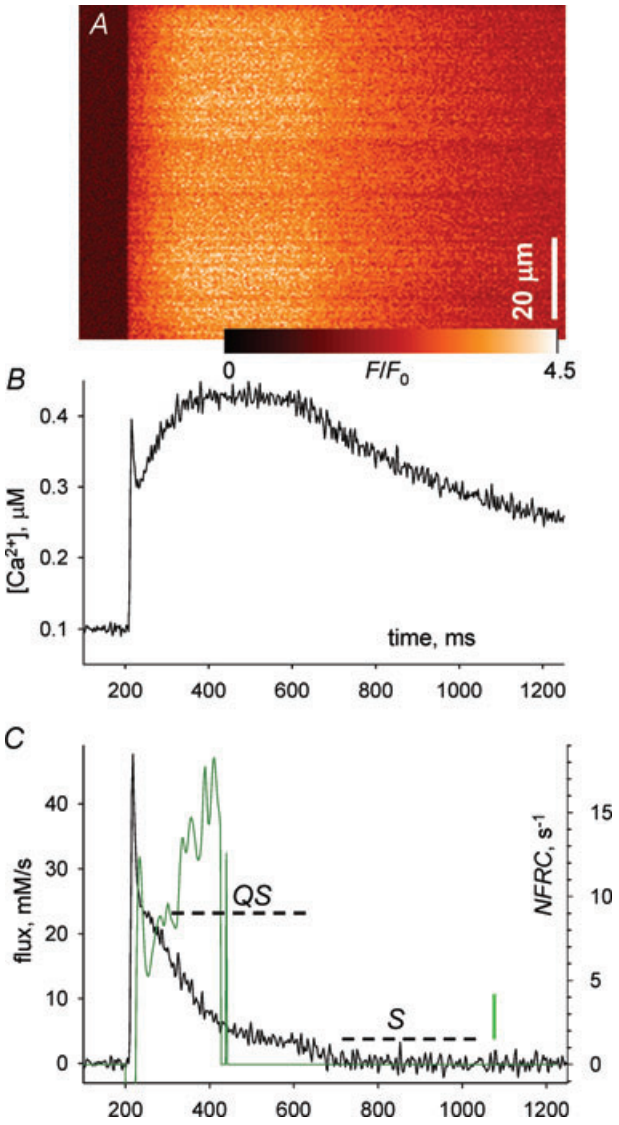

Figure 5. The *NFRC*, an index of non-exponential decay of release flux
A, line scan of rhod-2 fluorescence in a voltage-clamped mouse FDB cell stimulated by a 400 ms pulse to 0 mV. *B*, cytosolic $[Ca^{2+}](t)$ derived from the averaged line scan. *C*, black trace, release flux $\dot{R}(t)$ derived from the record in *B*, showing a 'shoulder' or sigmoidal decay, starting at level *QS* following the early peak and ending at steady level *S*. Green trace, *NFRC*(*t*), calculated according to equation in text. Note its steady growth during the time of the shoulder of flux. If flux decayed exponentially, *NFRC* would be constant. Calculation of *NFRC* is stopped when $\dot{R}_{net}$ becomes small compared with noise. Reproduced from Royer *et al.* (2008).

network to the channels constitute a pathway for enhanced delivery of Ca^{2+}. Right next to the channels the tendrils are likely to be single-stranded, and consequently most fragile. This link could break first upon depletion, leading to sudden changes in unitary release current.

As stated before, to reach beyond simple speculation, these ideas must be formulated within a quantitative framework, including size and density of the putative adsorption layer, defined kinetics of equilibration with the free solution, a formalism applicable to diffusion within this layer and an effective diffusion coefficient. No such framework can be built with the information now at hand, but there are feasible approaches that would help. One is to develop a test that can evaluate *in vitro* whether and to what extent the presence of polymerized calsequestrin favours diffusion of Ca^{2+}. Another is to combine the study of 'physiology' of individual cells and the evaluation of their Casq content. This could be started directly on wild-type muscles, which already provide a wide range of Casq content, as revealed for instance in anti-Casq1 immunostained images (Paolini *et al.* 2007, Fig. 2). A newly available double Casq-null mouse (obtained by mating the Casq1 and Casq2 mice) will make possible a number of experiments, from the simple comparison of its release flux

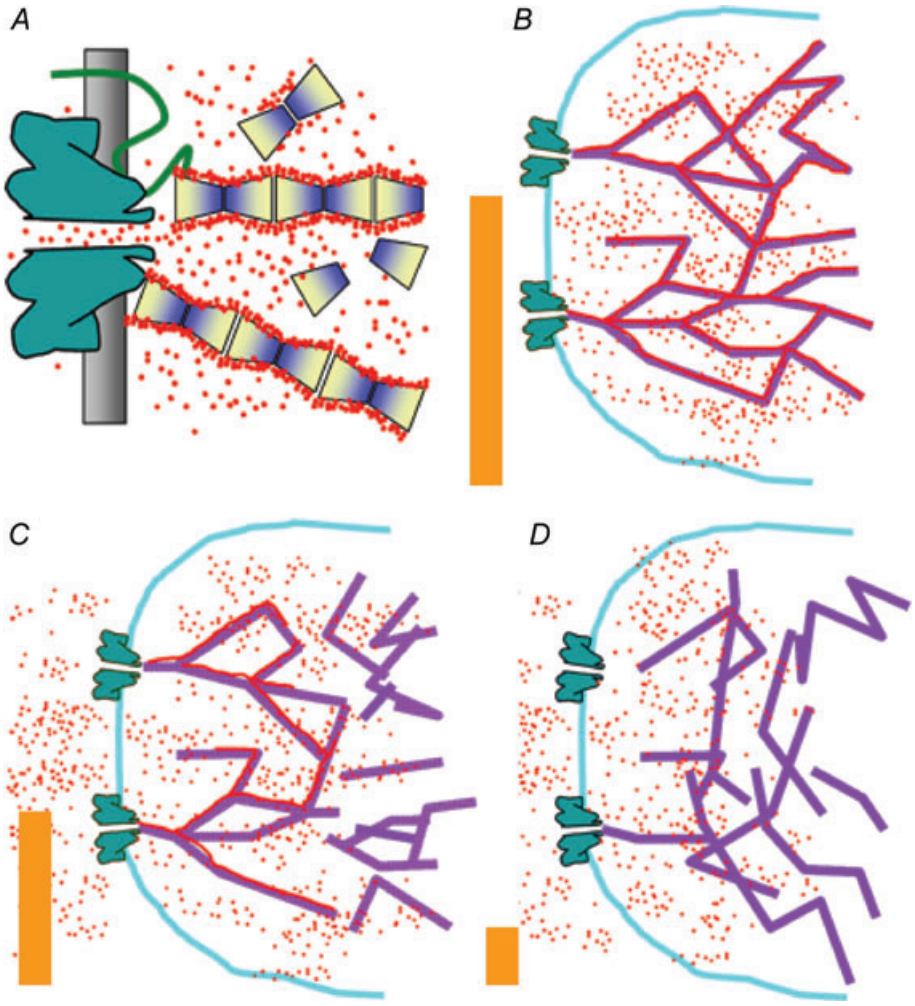

Figure 6. Aggregation-dependent buffering by calsequestrin?
A pictorial summary of ideas (MacLennan & Reithmeier, 1998; Park *et al.* 2003, 2004) which may help explain the observations. *A*, linear polymers of Casq1, with stereotyped alternation of front-to-front and back-to-back interactions, linked to the channel by triadin or junctin (green). Polymers feature a layer where Ca^{2+} ions (red) are adsorbed and may diffuse length-wise. These putative 'calcium wires', which copy structures visible in EM images (Fig. 1), link to a TC-wide network of Casq1 molecules that is not shown. *B–D*, sequential changes of the polymeric Casq1 network proposed to occur during Ca^{2+} release. Progressive depletion leads to de-aggregation. If the Ca^{2+} layer adsorbed on calcium wires is delivered to the channels more rapidly than dissolved Ca^{2+}, the buffering power *B* of the SR will decay as the Ca^{2+} wires are emptied or their connection to the channels collapse. The evolution of *B* is depicted by the height of the orange bar.

with that in the WT, to the study of flux in double Casq-null mice induced (by transfection of the adequate plasmid) to express Casq1 with its acidic C tail deleted. This preparation should lack the steps in *E*, as the equivalent mutation was shown to prevent aggregation-dependent buffering in solutions of Casq2 (Fig. 2*A*).

SR molecules other than Casq are able to buffer Ca^{2+} in large quantities

The work discussed in the previous sections indicates that calsequestrin plays an important role in supporting Ca^{2+} release and shaping its time course. Other observations, however, demonstrate that this role is not essential. A common finding of the recent work with Casq1-silenced skeletal myotubes, Casq1-null skeletal muscle fibres and Casq2-null cardiomyocytes is that these perform well in functional tests, including Ca^{2+} transients for myotubes and twitch and tetani for muscle fibres. As mentioned before, in some cases the estimates of SR Ca^{2+} content available for release did not find more than a small reduction, and even when they did (e.g. the study of Paolini *et al.* (2007), which found an 80% decrease in the integral of tension over time in a response to caffeine) there was sufficient releasable Ca^{2+} left to produce twitch and tetani of normal tension. The survival of the null animals and their modest functional compromise were unexpected in light of the well known fact that the endowment of calsequestrins greatly exceeds that of any other candidate Ca^{2+} ligand in the SR (e.g. Murphy *et al.* 2009, Fig. 3). For this reason, additional analyses have been carried out looking for increased expression of proteins or other changes that might compensate for the absence of calsequestrin.

The studies found in Casq1-null EDL muscle a near doubling of the content of RyR2 and increase in mitochondrial density, but no changes in SERCA or the 95 kDa triadin isoform, and a small reduction in Casq2 (Paolini *et al.* 2007). In C_2C_{12} myotubes it was found that the separate (virtually 100%) knockdown of each Casq isoform did not affect the level of expression of the other, while the contents of RyR (1 and 3) and SERCA1 were actually reduced by silencing of Casq2 or Casq1 and 2. None of seven other transport proteins studied were significantly changed (Wang *et al.* 2006; Meissner, 2009). In cardiac myocytes of Casq2-null mice the possible compensatory expression of other calcium binding proteins was sought with Western blots, ^{45}Ca overlays and Stains-all, but none was found (Knollmann *et al.* 2006; Chopra *et al.* 2007; Knollmann, 2009). SR volume increases, which should add minimally to the releasable Ca^{2+} as the majority of it should be bound rather than free in solution, were only found in the Casq2-null cardiomyocytes (a 50% increase). In summary, among the changes found in cells with calsequestrins reduced or ablated, the increase in RyRs (in the Casq1-null fast-twitch fibres) should provide a greater Ca^{2+} release permeability, and the increase in mitochondrial content could account for the increased resistance to fatigue. None of the changes could substantially replace lost capacity to store Ca^{2+}.

It continues to surprise us therefore, that muscle cells with total or partial deletion of Casq1 or 2 are still able to store and release large amounts of calcium. The quantitative contribution of calsequestrin to releasable Ca^{2+} storage may be less, as a fraction of the total, than it is generally believed to be. Other Ca^{2+}-binding proteins have been noted. Early work on rabbit SR fractions demonstrated substantial binding of $^{45}Ca^{2+}$ to extrinsic proteins that appeared in significant quantities in the extracts, including a '54 kDa component' from which calreticulin was later isolated and various 'acidic proteins' lighter than Casq (MacLennan *et al.* 1972).

In their commentary in 1998, MacLennan and Reithmeier noted that practically every known SR protein is very acidic, and suggested this to be a 'cost of doing business in a crowded and dangerous neighborhood' (MacLennan & Reithmeier, 1998). The proteins would use this feature both as protective gear and contribution to the common cause of storing Ca^{2+}. While much quantification is necessary to complete the picture, it seems that the neighbours can substitute fairly well for an absent calsequestrin.

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